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Loss of Dyes by HPLC Analysis in Ras Cheese and its Whey

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ABSTRACT

In a previous study, the extract of Turmeric and β-Carotene was extracted from local cheap products for coloring the Egyptian Ras cheese instead of the imported Annatto dye. Ras cheese was made with this extract, and compared with control cheese. HPLC technology was used to estimate the stability of the added dye during making of cheese when fresh) and during storage period (180 days), and compared with control samples. HPLC analyzed was performed to detect and follow the reduction of the colour of cheese, and the loss of dyes in the whey. Results showed that after extraction for HPLC analysis of the annatto extract (norbixin) used in making Ras cheese, the recovery of norbixin from fresh Ras cheese averaged 80.57%, while the norbixin content in the whey of Ras cheese was 19.35%. HPLC determination of β-Carotene in Ras cheese was about 4.28% from a total amount of β-Carotene being used in cheese making process is lost in whey. Recovery of β-carotene from fresh Ras cheese was 95.32%. Recovery of β-carotene from uncolored Ras cheese fresh Ras cheese and whey sample were (96.00%/3.50%), respectively. The results from HPLC determination of β-Carotene in ripened Ras cheese after six month old cheese were 37.23%. Residual Annatto was extracted from ripened Ras cheese after 180 days from storage which was 34.07%. While, residual curcumin percentage in Ras cheese after ripening time was 43.20%. Annatto is less stable compared to Curcumin and β-carotene extracts under the same condition. When used Curcumin and β-carotene extracts, they forming a stable colour little of which is lost during separation of the whey. The economic feasibility between the price of colorant added and loss of some of it in whey, it is clear that annatto is less expensive but will be larger loss of whey. In contrast, in the case of Curcumin and carotene extracts, a little amount of the added extract is lost in the whey. On the other hand, it is recommended that the waxing of Ras cheese wheels to preserve the colour of oxidative degradation.

Keywords: Annatto, β-carotene, HPLC, Curcumin.

INTRODUCTION

Colour in Ras cheese is also important factor affecting the acceptability of cheese, during green fodder feeding the cow milk had yellow colour especially from local Damietta cows, since imported cow produce yellow colour, as well the addition of 5-20% bright white buffaloes milk to cheese milk decrease the accepted yellow colour of the milk. Summer feeding excluded green fodder produces white cow milk. According to Hutchings (1994), the total appearance of a specific food product influences the individual's interpretation of all visually perceived information relating to that product. Besides smell and visually perceived flavour and texture, it is mainly the appearance properties, comprising optical properties, the physical form and the presentation mode (Hutchings, 1977), which are important in food choice (Rolls *et al*., 1982) and which contribute to sensory sensations and hedonic responses in the anticipation phase of consumption (Kramer, 1973). Several studies have shown that appearance attributes and, particularly, colour are associated with foodstuffs almost as frequently as flavour- and texture-related descriptors. It is, however, evident that the importance of appearance depends heavily on the type of food (Tuorila-Ollikainen *et al*., 1984 and Hamilton 1983). Schutz and Wahl (1981) reported on the high numbers of colour associations with fruits, vegetables and dairy products, whereas low frequencies were observed for flavour intensive liquids and cereals. Qualitative and quantitative sensory colour assessments have been frequently performed in order to monitor the effects of technological treatments on various traditional and processed cheese types.

Many Egyptian Ras cheese consumers thought that the high intensity yellow colour is related to the high fat content of the cheese. Cheese processor adding the available imported dye known as Annatto colorant. Annatto is the most important yellow-orange food colour additive widely used in the food industry particularly in the dairy and fat-based products to achieve a consistent colour over seasonal changes. Annatto's source is the outer coats of the seeds of the tropical Achiote trees (*Bixa orellana*) which native in North and South America, the Caribbean and the East Indies. Natural pigments have been used for a long time in many industries like food processing or cosmetic manufacturing. In Egypt, Annatto is unique in this regard especially in dairy products. Recently, the trend towards the use of natural colorant extracts have increased away from the traditional use due to the changing consumer culture towards more natural products, it also proved to play a functional role and provide a healthy benefit. On the other hand, there is a trend to enhance the utilization of food industry wastes to incorporate it again in food as a good source of bioactive compounds.

- One of these extract; Curcumin that's a natural compound isolated from rhizomes of the herb *Curcuma longa*, recognized for their broad spectrum of biological effects, therapeutic and pharmaceutical activities including anti-carcinogenic, immune response, anti-Alzheimer, anti-thrombotic, anti-inflammatory and anti-oxidant.
- Secondly, β-carotene extracted from carrot waste, β-carotene has a many positive health benefits such as pro-vitamin A, anti-oxidant, anti-cancer, Improve immunity and anti-inflammatory.

In a previous study was extracted some dyes of Curcumin from rhizomes of Curcuma longa and β-carotene from carrot peels, in an attempt to use them as an alternative to the colorant of the Annatto in Ras Egyptian cheese industry. Before quantifying and calculating percentages of pigments, these dyes must first be determined positively by using a method of HPLC, is the most common technique used for the determination of investigate Annatto, Curcumin and β-carotene followed by measuring their concentration in cheese and whey using an external standard curve prepared using the standard curve of the pure pigment of the extract used in the cheese synthesis separately (De Oliveira and Rodriguez-Amaya, 2007).

The aim of this paper was to investigate *Annatto*, *Curcumin* and β-*carotene* partitioning into Ras cheese and liquid whey using HPLC method to better understand Annatto behavior during the cheese making process and to investigate the viability of Curcumin and β-carotene as an alternative cheese coloring agents.

MATERIALS AND METHODS

Fresh cow and buffalo milks were obtained from the farm of Damietta, faculty of Agriculture. The chemical composition of milk used in Ras cheese making was: fat 5.1%, protein 3.8% and TS 14.7%. Dry fine commercial food grade salt was obtained from El-Nasr Company, Alexandria, Egypt.

All Materials and chemicals and Solvents were purchased from El-Gomhoria for chemicals company, Mansoura, Egypt.

Turmeric Rhizomes (*Curcuma longa*) was purchased from herbal market in Damietta, Egypt.

Carrot was obtained from local market, Damietta, Egypt.

Curcumine Crystalline extra pure (assay 98%) was purchased from Alpha Chemika, India.

Pure Beta carotene powder with a minimum purity of 99% purchased from Alpha Chemika, India, and commercial Liquid annatto (assay 4%) was purchased from MIFAD Company, Badr City Egypt.

Curcumin extraction was carried out as described by Joshi *et al*. (2009); Kulkarni *et al*. (2012) and Mainum & Shashikala (2014), with some modifications:

The rhizomes of turmeric were dried in a hot air oven at 50oC for even access to a constant weight, ground to a fine powder by electronic mill powder and passed through a sieve (40 mesh). The extract was prepared by extracting 5gm of the turmeric powder packed in a filter paper to allow the solvent reaching to the sample with (250ml) ethanol 95% using a Soxhlet's extraction apparatus for 4 hours at 60°C or till the solvent in siphon tube of an extractor become colorless. The extract was filtered by using filter paper was put into a funnel and placed over a Buchner flask that was attached to a vacuum. The clear filtrate was concentrated on rotary evaporator an even access to a third of the volume. The remaining amount has been distributed in Petri dashes known weight and dried in oven at 40C even the weight is stable, these steps are summarized in Figure (1).

Carrot peels powder was prepared by washing carrot with warm water (30ºC), hand peeled; then it was dried at (60ºC for 12hr) in an electric oven drier, milled using an electric mill to a particle size of 500–600µm to pass through 20 mesh sieve, packed in polyethylene bags and stored in refrigerator until use.

Beta-carotene extraction was carried out as described by Fikselova *et al.* (2008); Wingqvist (2011) and Sharmin *et al.* (2016) with some modifications; carrot waste is used as a source of beta-carotene. The extraction procedures are summarized in Figure (2), where it will be extracted by using of fine carrot peels powder and Soxhlet extraction method. Carrot peels powder was prepared by washing carrot with tap water, hand peeled, freezing (–5°C), then it was dried at (60ºC for 12hr) in an electric oven drier, milled using an electric mill to a particle size of 500–600µm to pass through 20 mesh sieve, packed in polyethylene bags and stored in refrigerator until use. 5 grams fine carrot peels powder is placed in a filter paper to allow the solvent reaching to the sample. The round boiling flask is filled with 250ml of solvent ethanol 95%. The extraction process is performed at 60°C, lasted about 4 hours until adsorbent became colorless Figure (2). The extract was filtered by using filter paper Whattman No. (1) was put into a funnel and placed over a Buchner flask that was attached to a vacuum. The clear filtrate was concentrated on rotary evaporator an even access to a third of the volume. The remaining amount has been distributed in Petri dashes information weight and dried in oven at 40°C even the weight stability. After extraction, the size of the extract is concentrated to 50mL, which is used to estimate beta-carotene by spectrophotometer.

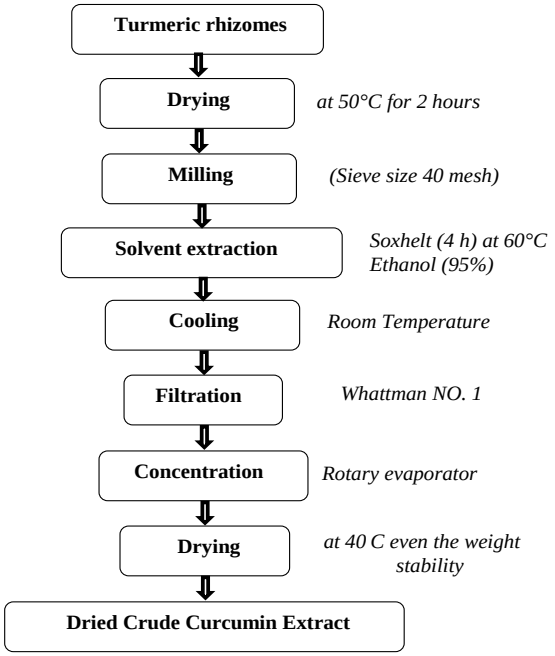

Figure 1. Extraction of *Curcumin* from *Turmeric rhizomes (Curcuma longa)*

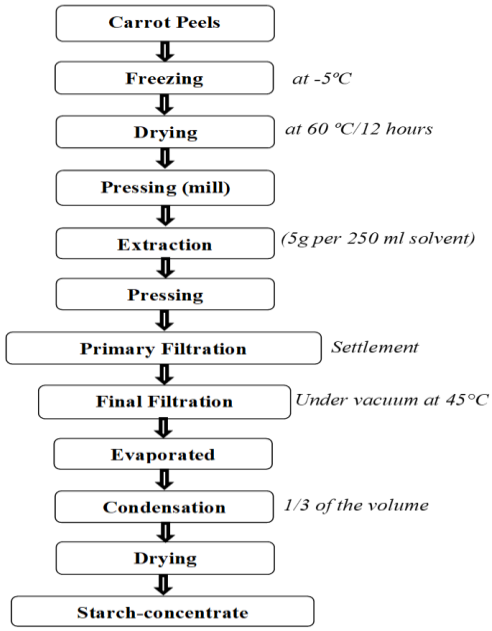

Figure 2. Extraction of beta-carotene from Carrot peels

Experimental design:

- Ras cheese as control and samples were manufactured in El-Faiomy laboratory, Damietta for making Ras cheese.
- Four treatments were performed for the manufacture of Ras cheese (Figure 3). A total of 120 L of raw Standardized milk was used and divided into batches by changing cheese colour, the treatments were as following:

1. **(T0) NRC: (None colorant Ras cheese)**: Ras cheese is made without adding any colorant.
2. **(T1) ARC: Annatto Ras cheese (commercial Ras cheese):** Ras cheese is made with Annatto (0.03% (v/v)).
3. **(T2) CRC: Curcumin Ras cheese:** Ras cheese is made with Curcumin extract (0.03% (v/v)).
4. **(T3) BRC: Beta-carotene Ras cheese:** Ras cheese is made with beta-carotene extract (0.03% (v/v)).

- Samples for analysis were periodically taken at 0, 15, 30, 60, 90, 120,150 and 180 days of ripening.

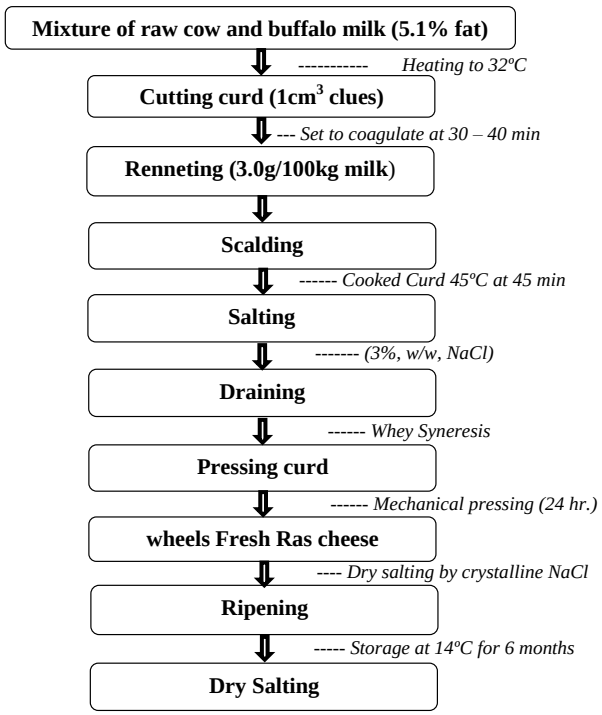

Figure 3. The flow chart for manufacturing Ras cheese

Determination of *Annatto*, *Curcumin* and β-*Carotene* in Ras cheese and whey:

The extraction procedures for HPLC of the cheese and whey were carried out as described by Scotter *et al*. (2002). The concentrated extract was quantitatively transferred to a 25 ml volumetric flask with methanol and diluted to the mark. After mixing, a 2-ml aliquot was transferred to a glass syringe fitted with a micro-filter (0.2 mm) and subsequently filtered into a vial for analysis by HPLC.

RESULTS AND DISCUSSION

Partitioning of annatto, Curcumin and β-carotene in the Ras cheese and its whey:

Generally, in order to give the desirable yellow colour of hard cheese, annatto is added into milk vat during cheese making process, and some of the annatto is also lost in whey (Smith *et al*., 2014). This yellow colour is unfavorable (Chang *et al*., 1977). However, it is desired in Cheddar cheese, but the whey has a neutral colour (Campbell *et al*., 2011).

After extraction procedures for HPLC analysis in the presnt work for the commercial Annatto extract (norbixin) in Ras cheese, the recovery of norbixin from fresh Ras cheese averaged 80.57%, while the norbixin, obtained for the whey of Ras cheese was 19.35%. The HPLC chromatograms (Figure 4 and 5) obtained from the fresh of Ras cheese with annatto and its whey. These results are consistent with a previous study conducted by Kang *et al*. (2010). Croissant *et al*. (2009) stated that norbixin is usually used because it is soluble in water and dispersible, it is often assumed that norbixin in whey is present mainly in the serum phase (Giuliano *et al*., 2003). However, (Govindarajan and Morris, 1973 and Hammond *et al*., 1975) suggested that norbixin in whey might be tightly bound to whey proteins that it binds to the caseins of Cheddar cheese curd.

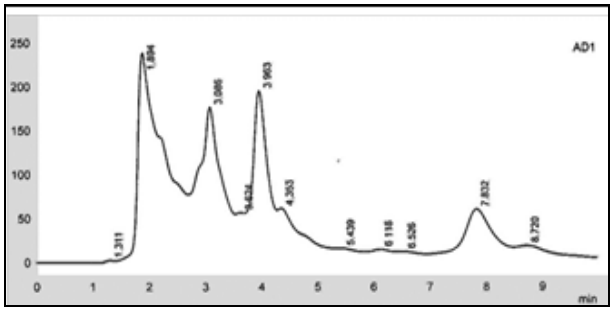

Figure 4. A chromatogram obtained from the Ras cheese curd with annatto

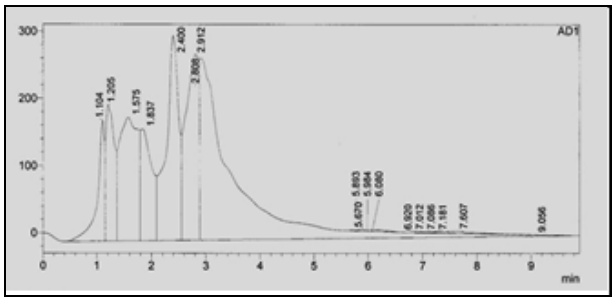

Figure 5. A chromatogram obtained from the whey Ras cheese with annatto

β-*Carotene* distribution between Ras cheese and whey:

The diet of the cow can influence the color of cheese β-carotene representing about 90% of total carotenoids present in cow's milk. In ripened cheese without added colorant, carotenoid transfer to cheese and also carry over into fluid whey during cheese making process analysed cheddar cheese made with β-carotene colorant. Recovery of β-carotene was 109%, the recovery rate for b-carotene was thought to be enhanced by the presence of a significant level of naturally occurring b-carotene. For these reasons it was necessary to estimate beta-carotene in the samples of whey and Ras cheese made from raw milk without adding any color to avoid the calculation error when estimated in the samples of the Ras made from raw milk with adding the β-carotene extract. Samples with no colorant as control and samples with β-carotene were similarly processed. (Scotter *et al*. 2003, Carpino *et al*., 2004, Noziere *et al*., 2006, Hulshof *et al*., 2006 and Kang *et al*., 2010).

Figures (6-9) show the HPLC chromatograms obtained from fresh Ras cheese and whey made with the addition of coloring agents, and fresh Ras cheese made from raw milk without adding any colour. HPLC quantification was carried out with the aid of a calibration line by standard β-carotene. The results from HPLC determination of β-Carotene in Ras cheese showed about 4.28% from a total amount of β-Carotene is used in cheese making process is lost in whey. Recovery of β-carotene from fresh Ras cheese was 95.32%. Recovery of β-carotene from uncolored Ras cheese fresh Ras cheese and whey sample were (96.00%/3.50%), respectively. These results came in agreement with (Chapman *et al*. 1980, Zahar *et al*., 1995, and Lucas *et al*., 2006 and Campbell *et al*. 2012).

***Curcumin* distribution between Ras cheese and whey:**

The percentage of Curcumin recovered from fresh Ras cheese was 83.79%. Residual Curcumin was extracted from liquid whey which was 16.20%.

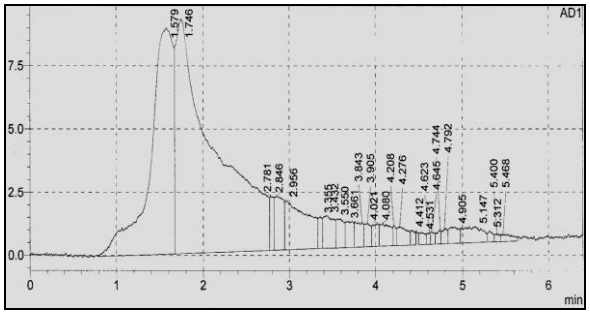

Figure 6. A chromatogram obtained from the fresh Ras cheese with β-carotene

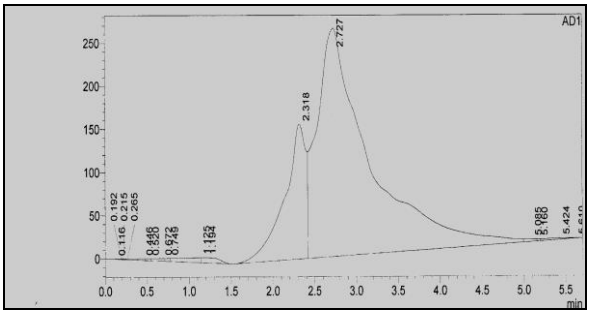

Figure 7. A chromatogram obtained from the whey Ras cheese with β-carotene

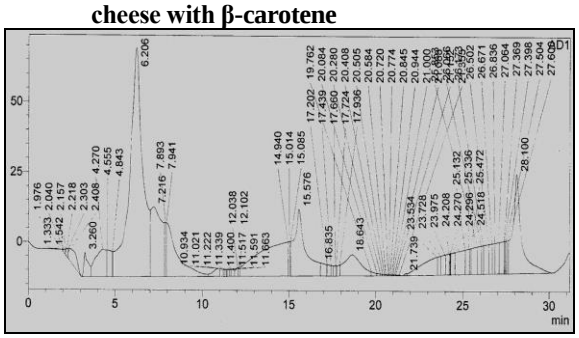

Figure 8. A chromatogram obtained from the fresh Ras cheese without colorant added

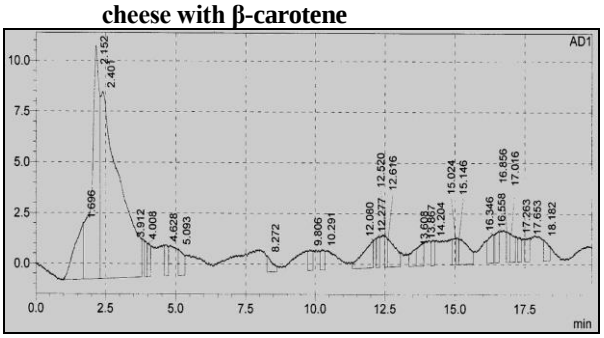

Figure 9. A chromatogram obtained from the whey Ras cheese without colorant added

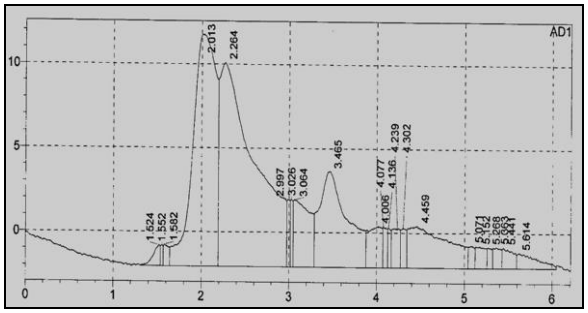

Figure 10. A chromatogram obtained from the fresh Ras cheese with *Curcumin*

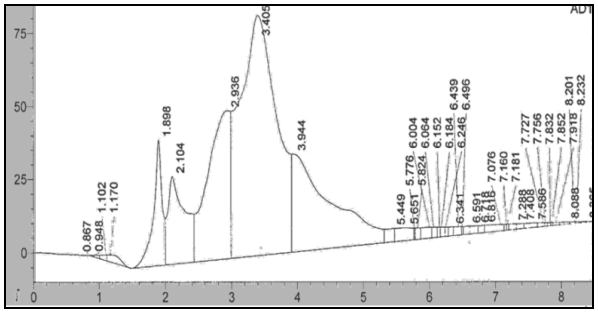

Figure 11. A chromatogram obtained from the whey Ras cheese with *Curcumin*

Stability of the Annatto, Curcumin and β-carotene dyes as affected by ripening period of Ras cheese wheels

The stability of dyes added to cheese during ripening period is the most important standard especially in terms of quality and sensory properties. The degradation of norbixin, curcumin and β-carotene were monitored by high-performance liquid chromatography (Figures 12-15).

The results from HPLC determination of β-Carotene in ripened Ras cheese after six month old cheese were 35% and 37.23% for treatments of cheese without colour added and β-carotene cheese respectively. Residual Annatto was extracted from ripened Ras cheese after 180 days from storage which was 34.07%. Therefore, it is preferable to consume cheese without any colorant added freshly and not ripened it for long periods. While, residual curcumin percentage in Ras cheese after ripening time was 43.20%. Mortensen *et al*. (2004) reported that Pink discoloration in cheeses during storage caused by photooxidation, which affected by properties of light, storage temperature, time of storage, type of colorants used and pH of cheese. The results showed that Annatto and β-*carotene* levels in the ripened Ras cheese after 180 days of storage markly decreased compared with *curcumin* cheese. The light can be more destructive to carotenoids than *curcumin*. Both Annatto and β-*carotene*, as a carotenoid, is sensitive to oxidation in foods (Hong *et al*., 1995 and Smith, 2004). Norbixin have highly conjugated structures, this leads to susceptibility to oxidation (Giuliano *et al*. 2003 and Smith 2004). And similarly, β-*carotene* is a very reactive compound due to its highly unsaturated structure, which renders it electronically rich by delocalisation of electrons. Consequently, it is also prone to degradation and more precisely to isomerisation, especially at high temperature, and oxidation, due to the occurrence of oxygen in food (Penicaud *et al*., 2011). Leading to

degradation with concomitant colour changes and loss of colour intensity. On the other hand, the percentage of β-carotene loss is relatively lower compared to Annatto treatment, where it was 37.23% and 34.07%, respectively.

Similar observations were found by Petersen *et al*., (1999), where the decrease in colour intensity was significantly higher for the cheddar cheeses colored with *annatto* than for those colored with β-*carotene*. similar trends were reported by Craig (2007), who reported that *annatto* was the least stable when comparing stability of some carotenoids as a colorant in Process Cheese Spread, Where the order according to stability was as follows β-APO-8'-carotenal=Paprika Oleoresin> Astaxanthin > β-*carotene* >*Annatto*. On the other hand, Daly *et al*. (2012) indicated that the defect of pink cheese with annatto was developed due to decrease in cheese pH < 5.4 may lead to the precipitation of norbixin, which is associated with phospholipid. Also, norbixin binds with divalent cations such as calcium and form its salts leading to development of pink precipitate. While, in the case of *Curcumin*, acidic pH is considered to be the most important factor on the stability of *Curcumin* during food storage, it is most stable in the pH range (1–6) as reported by (Tonnesen and Karlsen, 1985 and Goel *et al*., 2008).

The results are interesting because they indicate that Annatto is less stable compared to Curcumin and β-carotene extracts under the same condition, although it is popularly used in many food and dairy products. When used Curcumin and β-carotene extracts, they forming a stable colour little of which is lost during separation of the whey. The economic feasibility between the price of colorant added and loss of some of it in whey, it is clear that annatto is less expensive but will be larger loss of whey. In contrast, in the case of Curcumin and carotene extracts, a little amount of the added extract is lost in the whey. On the other hand, it is recommended that the waxing of Ras cheese wheels to preserve the colour of oxidative degradation.

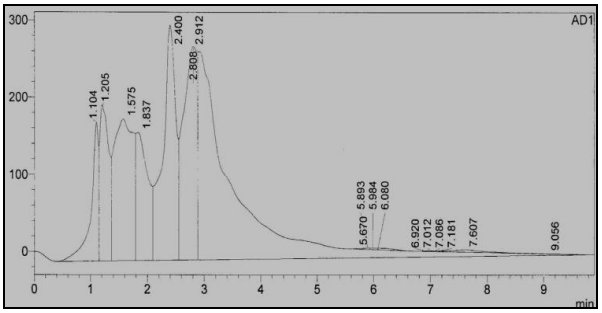

Figure 12. A chromatogram obtained from the ripened Ras cheese with β-*carotene*

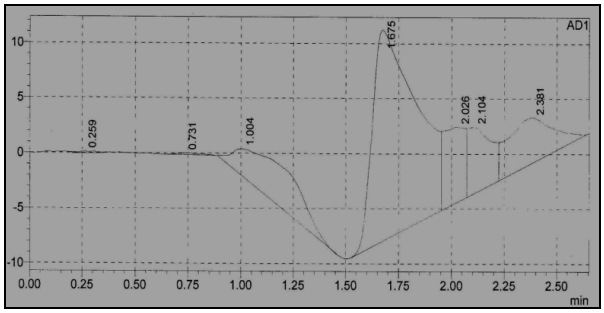

Figure 13. A chromatogram obtained from the ripened Ras cheese with *Annatto*

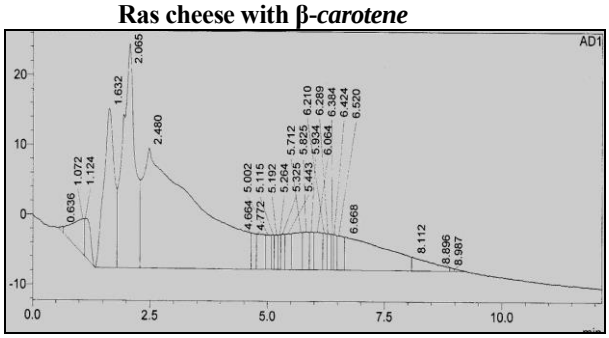

Figure 14. A chromatogram obtained from the ripened Ras cheese without colorant added

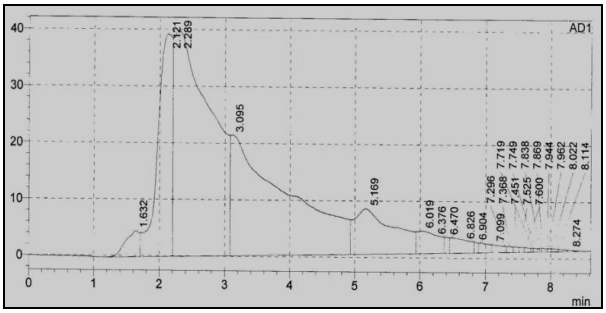

Figure 15. A chromatogram obtained from the ripened Ras cheese with *Curcumin*

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تقدير فقد الصبغات بتقنية التحليل الكروماتوجرافي السائل العالي الكفاءة HPLC للجبن الراس والشرش

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في دراسة سابقة، تم إستخراج مستخلص الكركم والبيتاكاروتين من المنتجات المحلية الرخيصة لإستخدامها في تلوين الجبن الراس بديلاً عن صبغة الأناتو المستوردة, وتم تصنيع جبن راس بهذه المستخلصات ومقارنتها مع الجبن المصنع بالأناتو, تم إستخدام تقنية HPLC لتقدير ثبات الصبغة المضافة بعد التصنيع (طازجة) وبعد فترة التخزين (180 يومًا) ومقارنتها بعينات الكونترول, تم التحليل بجهاز HPLC لكشف ومتابعة تناقص لون الجبن، وكذلك الأصباغ المفقودة الموجودة في مصل اللبن المختلف, وخلصت النتائج إلى ما يلي:تم إستخراج مستخلص الأناتو التجاري (نوربيكسين) المستخدم في الجبن الراس بتقنية الـ HPLC، وبلغت نسبة إسترداد النوربيكسين من الجبن الراس الطازجة 80.57٪ ، بينما بلغت نسبة النوربيكسين في الشرش 19.35٪.أظهرت نتائج تقدير HPLC للبيتا كاروتين أن حوالي 4.28٪ من إجمالي كميته المستخدمة في عملية تصنيع الجبن تضيع في الشرش, وكان إسترداد البيتاكاروتين من الجبن الطازج 95.32٪, ومن الجبن الطازج غير المملح والشرش (96.00 ٪ / 3.50 ٪)، على التوالي. كانت نسبة البيتاكاروتين في الجبن المخزن بعد ستة أشهر تبلغ 37.23 ٪, بينما كانت نسبة الأناتو 34.07٪, في حين بلغت نسبة الكركمين المتبقية في الجبن الراس كانت 43.20٪. النتائج تشير إلى أن صبغة الأناتو أقل استقرارًا مقارنة بمستخلصات الكركمين والبيتاكاروتين في نفس الحالة. عند استخدام مستخلصات الكركمين والبيتاكاروتين تعطي للمنتج لونًا ثابتًا يضيع القليل منه أثناء فصل الشرش. الجدوى الاقتصادية تميل إلي إستخدام الملونات غير الأناتو بالرغم من أن الأناتو أقل في التكلفة ولكنه يُفقد في الشرش بصورة أكبر عن مستخلصات الكركمين والبيتاكاروتين.